Interleukin-4 inhibition of osteoclast differentiation is stronger than that of interleukin-13 and they are equivalent for induction of osteoprotegerin production from osteoblasts

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Summary

Interleukin (IL)-4 and IL-13 are closely related cytokines known to inhibit osteoclast formation by targeting osteoblasts to produce an inhibitor, osteoprotegerin (OPG), as well as by directly targeting osteoclast precursors. However, whether their inhibitory actions are the same remains unclear. The inhibitory effect of IL-4 was stronger than that of IL-13 in an osteoclast-differentiation culture system containing mouse osteoblasts and osteoclast precursors. Both cytokines induced OPG production by osteoblasts in similar time- and dose-dependent manners. However, IL-4 was stronger in direct inhibition that targeted osteoclast precursors. Furthermore, IL-4 induced phosphorylation of signal transducer and activator of transcription-6 (STAT6) at lower concentrations than those of IL-13 in osteoclast precursors. IL-4 but not IL-13 strongly inhibited the expression of nuclear factor of activated T-cells, cytoplasmic 1 (nuclear factor-ATc1), a key factor of osteoclast differentiation, by those precursors. Thus, the activities of IL-4 and IL-13 toward osteoclast precursors were shown to be different in regards to inhibition of osteoclast differentiation, whereas those toward osteoblasts for inducing OPG expression were equivalent.

Keywords: IL-4; IL-13; osteoclasts; osteoblasts; cellular differentiation

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Introduction

Osteoclasts are multinucleated giant cells that play a role in bone resorption during bone metabolism. Osteoclast differentiation is induced by the receptor activator of nuclear factor (NF)-κB ligand (RANKL), which is produced by osteoblasts/stromal cells.[1] Osteoblasts/stromal cells also produce osteoprotegerin (OPG), a decoy receptor for RANKL, which inhibits osteoclast differentiation by interrupting the interaction between RANKL and its receptor RANK.[2–4] OPG gene-deficient mice exhibit severe osteoporosis, because of the increase in number of osteoclasts.[5,6] Therefore, a balanced gene expression level of RANKL and OPG is critical for regulation of bone mineral density.

Many investigators have explored the mechanisms of osteoclast differentiation using coculture and bone marrow-derived macrophage (BMM) culture systems. In

Abbreviations: IL, interleukin; OPG, osteoprotegerin; BMC, bone marrow cell; BMM, bone marrow-derived macrophage; RANKL, receptor activator of nuclear factor κB ligand; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1.

previous coculture systems, bone marrow cells (BMCs) and osteoblasts were cocultured in the presence of 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], which induced the production of RANKL and suppressed that of OPG in osteoblasts.[7,8] Further, in a BMM culture system, BMMs that were shown to be identical to osteoclast precursors were cultured in the presence of M-CSF and RANKL proteins.[9] Although both culture systems produced osteoclasts, the coculture system is considered to be more effective for examining the role of osteoblasts in osteoclast differentiation, while the BMM culture system is useful to examine the direct actions of various factors toward osteoclast precursors.

It has been reported that lymphocyte-derived cytokines play critical roles during bone metabolism under physiological and pathological conditions.[10] IL-4 and IL-13, T helper 2 (Th2) cytokines, are pleiotropic lymphokines produced by antigen activated T cells that play particularly important roles in inflammatory and immune responses.[11] Because IL-4 and IL-13 share a common receptor complex, quite similar intracellular signals are activated by these cytokines.[10,12] In addition, binding of IL-4 to the receptor IL-4Rα induces heterodimerization with a common γ-chain (γc) (type I receptor complex) or IL-13Rα1 (type II receptor complex). On the other hand, binding of IL-13 to its receptor IL-13Rα1 with moderate affinity induces heterodimerization with IL-4Rα. Such dimerization of the receptors activates downstream signalling molecules, including Janus kinase-1 (JAK1) and -3, and signal transducer and activator of transcription-6 (STAT6).[12] However, the expression levels of the receptors for IL-4 and IL-13 are regulated differently depending on cell type.

IL-4 is known to suppress RANKL-induced osteoclast differentiation through direct action on osteoclast precursors.[13] Recently, Palmqvist *et al.* reported that both IL-4 and IL-13 inhibited osteoclast differentiation by a mechanism that increased OPG in osteoblasts, and also decreased RANKL and RANK expression.[14]

In the present study, we compared the effects of IL-4 and IL-13 toward the inhibition of osteoclast differentiation using both coculture and BMM culture systems. Our results suggest that IL-4 is more effective than IL-13 toward osteoclast precursors, while they are equivalent in regards to stimulation of OPG production from osteoblasts.

Materials and methods

Chemicals

Recombinant murine IL-4 and IL-13, and transforming growth factor-β (TGF-β) were purchased from R & D systems (Minneapolis, MN). Alpha-modified minimum essential medium (α-MEM) and 1α,25$(OH)_2D_3$ were purchased from Sigma (St. Louis, MO). Human macrophage colony-stimulating factor (M-CSF, Leukoprol®) was purchased from Kyowa Hakko Kogyo (Osaka, Japan). The soluble form of human TRANCE (human RANKL) was a kind gift from Dr Yongwon Choi (University of Pennsylvania School of Medicine).[15,16]

Cell cultures

Primary calvarial osteoblasts were obtained from the calvariae of neonatal ddY mice (Saitama Experimental Animals, Saitama, Japan) using 0·1% collagenase and 0·2% dispase.[17] BMCs were obtained from the long bones of 4- to 6-week-old ddY male mice. To obtain BMMs, BMCs were cultured for 3 days in α-MEM containing 10% fetal bovine serum (Sigma), M-CSF (50 ng/ml), and TGF-β (1 ng/ml) in 100-mm diameter type-I collagen-coated culture dishes (IWAKI-Asahi Glass, Tokyo, Japan) (1×10^7 cells/dish). After culturing for 3 days, cells attached to the culture plates were collected and used as BMMs. In the BMM culture system, BMMs were cultured on 96-well cell culture plates (Corning, Corning, NY) in the presence of M-CSF (50 ng/ml) and RANKL (150 ng/ml) for 4 days. In the coculture system, BMMs (2×10^4 cells/well) or BMCs (1×10^5 cells/well) were cultured on 96-well cell culture plates (Corning) with mouse primary osteoblasts or cells from the stromal/osteoblastic cell line UAMS-32 (5×10^3 cells/well), in the presence or absence of 1α,25-$(OH)_2D_3$ (10 nM) for 6 days. After culturing, some of the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts.

Measurement of TRAP activity

Cells in a 96-well culture plate were rinsed with phosphate-buffered saline and dissolved with 150 μl of lysis buffer (50 mM acetic acid buffer, pH 5·0, containing 1% sodium tartrate and 0·1% Triton-X). The cell lysates were briefly sonicated, then 20 μl of cell lysate was mixed with 100 μl of *p*-nitrophenyl phosphate solution (1 mg/ml in 50 mM acetic acid buffer, pH 5·0, containing 1% sodium tartrate) and incubated at 37° for 30 min. After the addition of 50 μl of 1 M NaOH, absorbance was measured at 405 nm.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA (1 μg) was reverse-transcribed using Superscript II (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocols. PCR was performed using Taq DNA polymerase (Sigma) under the following conditions: denaturation at 94° for 30 s, annealing at 58° for 30 s, and extension at 72° for 30 s, with

25 cycles for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 30 cycles for mouse IL-4Rα, γ_c, IL-13Rα1, and NFATc1. The oligonucleotide primers used for RT–PCR were as follows:

GAPDH (452 bp), 5′-GAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′; mouse IL-4Rα (432 bp), 5′-ATCTGCGTGCTTGCTGGTTCT-3′ and 5′-CTGGTATCTGTCTGATTGGACCG-3′; mouse γc (266 bp), 5′-GTTCTGAGCCTCAGGCAACC-3′ and 5′-CAGATTGCTGAGTGTTAGAT-3′; mouse IL-13Rα1 (385 bp), 5′-CATCTTCTCCTCAAAAATGGTGCC-3′ and 5′-GGATTATGACTGCCACTGCGAC-3′; and mouse NFATc1 (565 bp), 5′-TCATCCTGTCCAACACCAAA-3′ and 5′-TTGCGGAAAGGTGGTATCTC-3′.

Northern blot analysis

Ten micrograms of total RNA was extracted from the cells and electrophoresed on a 1% agarose gel containing 0·22 M formaldehyde, then blotted onto nylon membranes (Hybond-N, Amersham Biosciences, Amersham, UK). Filters were serially hybridized with cDNA probes for mouse OPG, RANKL, and 18S rRNA, then hybridization was performed in Perfect Hybridization Solution (Sigma) overnight at 65°. The membranes were washed twice with 2× saline sodium citrate (SSC) containing 0·1% sodium dodecyl sulphate (SDS) at 65° for 30 min and once with 2× SSC at 65° for 30 min, followed by exposure to a storm phosphoimager screen (Molecular Dynamics, Sunnyvale, CA). Signals were visualized and quantitated using ImageQuant software (Molecular Dynamics).

Western blot analysis

Western blot analysis was performed as described previously.[18] Briefly, the lysates of BMMs and UAMS-32 cells treated with various concentrations of IL-4 and IL-13 were subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were incubated with antibodies against murine STAT6 and phospho-STAT6 (Cell Signaling Technology, Beverly, MA), following incubation with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technologies). Protein bands were detected by use of ECL plus Western Blotting Detection system (Amersham Biosciences) and exposed to a FUJI medical X-ray film (FUJIFILM, Kanagawa, Japan).

Quantification of OPG

Cells were cultured for various periods with increasing amounts of OPG. The amount of OPG released into the culture medium was determined using a mouse OPG/TNFRSF11B Immunoassay kit (R & D Systems, Inc.) according to the manufacturer's instructions.

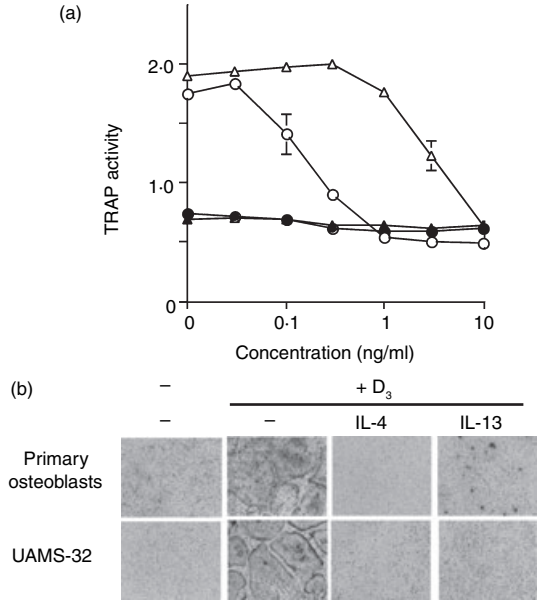

Figure 1. Effects of IL-4 and IL-13 on osteoclast differentiation in a coculture system. (a) BMMs were cocultured with UAMS-32 cells and various concentrations of IL-4 (○) or IL-13 (△) in the presence of 100 nM 1α,25-$(OH)_2D_3$. The same cultures were also performed in the absence of 1α,25-$(OH)_2D_3$ (D_3) with IL-4 (●) or IL-13 (▲). After culturing the cells, cell lysates were harvested and TRAP activity was measured. Data are shown as the means ± SE ($n = 4$). (b) BMMs cocultured with primary osteoblasts or UAMS-32 cells in the absence or presence of 10 nM of 1α,25-$(OH)_2D_3$ (D_3), 10 ng/ml of IL-4 or 10 ng/ml IL-13. The cells were fixed and stained for TRAP (original magnification: ×10).

Results

Inhibition of osteoclast differentiation by IL-4 and IL-13 in coculture system

We first examined the effects of IL-4 and IL-13 on osteoclast differentiation in a coculture system, in which mouse BMMs were cultured with UAMS-32 cells in the presence of 1α,25-$(OH)_2D_3$, an inducer of RANKL expression. The addition of IL-4 at a concentration of 1 ng/ml to the cocultures completely inhibited osteoclast differentiation, whereas 10 ng/ml of IL-13 was required for complete inhibition (Fig. 1a, b). The IC_{50} values of IL-4 and IL-13 for osteoclast differentiation were 0·13 and 2·69 ng/ml, respectively (Fig. 1a). These results suggest that the inhibitory activity of IL-4 is stronger than that of IL-13 in the coculture system.

Regulation of OPG and RANKL expression in osteoblasts by IL-4 and IL-13

Because OPG produced by osteoblasts is a potent inhibitor of osteoclast differentiation, we examined the expression levels of OPG mRNA before and after treatment

with IL-4 or IL-13 in UAMS-32 cells. Both cytokines increased OPG mRNA expression levels in similar dose- and time-dependent manners (Fig. 2a, b). Similarly, OPG protein levels in the culture supernatants of UAMS-32 cells were also increased following treatment with IL-4 and IL-13 in dose- and time-dependent manners (Fig. 2c, d). IL-4 and IL-13 have been shown to recover OPG mRNA expression down-regulated by 1α,25-$(OH)_2D_3$ in mouse calvariae and osteoblasts,[14] therefore we also examined their effects on OPG mRNA expression levels in the presence of 1α,25-$(OH)_2D_3$. Addition of the cytokines led to recovery of the down-regulation of OPG gene expression by 1α,25-$(OH)_2D_3$ (Fig. 2e). Further, the expression levels of RANKL mRNA were decreased in a similar dose-dependent manner (Fig. 2f). These results suggest that IL-4 and IL-13 act in a similar manner toward osteoblasts to stimulate the production of OPG, which inhibits osteoclast differentiation.

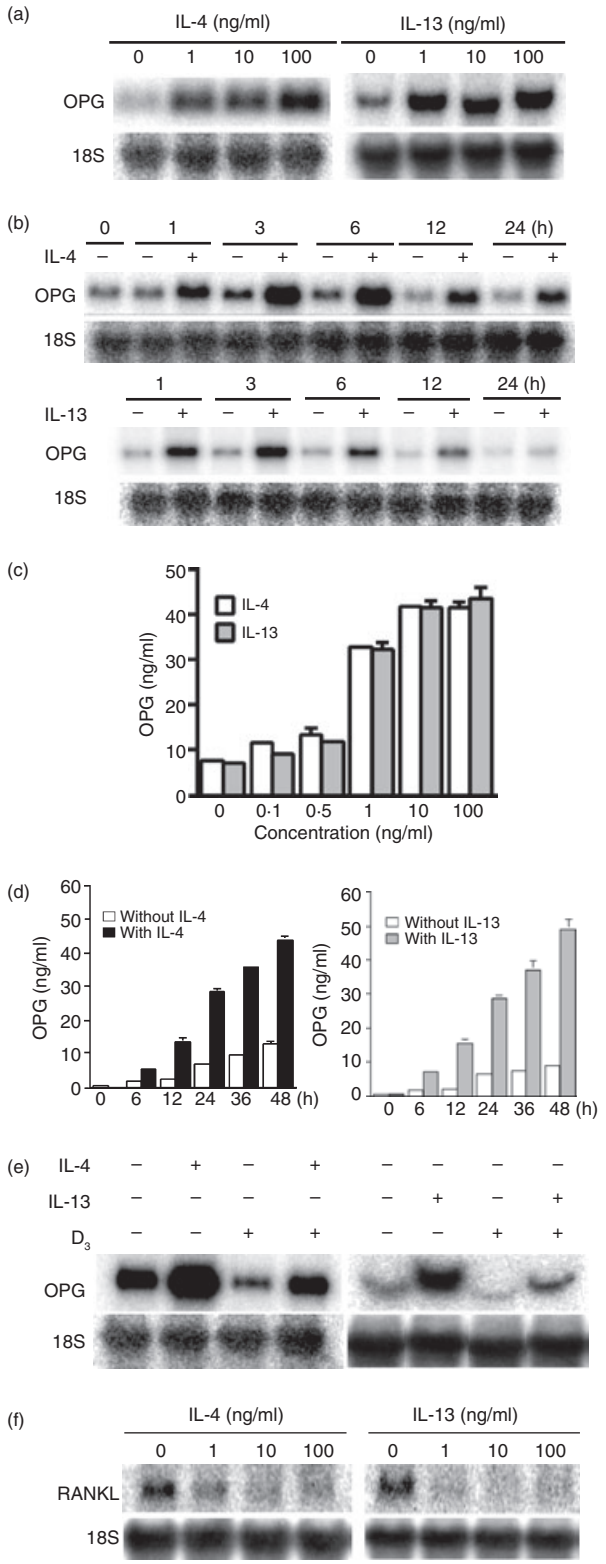

Figure 2. Regulation of OPG and RANKL gene expression by IL-4 and IL-13 in osteoblastic cells. (a) Dose effects of IL-4 and IL-13 on the induction of OPG mRNA. UAMS-32 cells were treated with 1, 10, and 100 ng/ml of IL-4 or IL-13 for 3 hr. (b) Time course analysis of OPG mRNA expression in the presence of IL-4 or IL-13. UAMS-32 cells were treated with 10 ng/ml of IL-4 or IL-13 for 1, 3, 6, 12, and 24 hr. (c) Dose effects of IL-4 and IL-13 on the secretion of OPG protein. UAMS-32 cells were treated with 0·1, 0·5, 1, 10, and 100 ng/ml of IL-4 (white bars) or IL-13 (grey bars) for 12 hr. Data are shown as the means ± SD ($n = 3$). (d) Time course analysis of the secretion of OPG protein in the presence of IL-4 or IL-13. UAMS-32 cells were treated with 10 ng/ml of IL-4 (black bar) or IL-13 (grey bar) for 6, 12, 24, 36, and 48 hr. Concentrations of OPG in the conditioned media of UAMS-32 cells were determined by enzyme-linked immunosorbent assay. Data are shown as the means ± SD ($n = 3$). (e) Effects of IL-4 and IL-13 on OPG mRNA in osteoblastic cells pretreated with 1α,25-$(OH)_2D_3$. UAMS-32 cells were pretreated with 100 nM of 1α,25-$(OH)_2D_3$ for 1 hr, followed by treatment with 10 ng/ml of IL-4 or IL-13 for 3 hr. (f) Effects of IL-4 and IL-13 on RANKL mRNA in osteoblastic cells. UAMS-32 cells were treated with 1, 10, and 100 ng/ml of IL-4 or IL-13 for 24 hr. (a, c, e, and f) The expressions of RANKL mRNA, OPG mRNA, and 18S rRNA were detected by Northern blot analysis.

Inhibition of RANKL-induced osteoclast differentiation by IL-4 and IL-13

We next examined the direct effects of IL-4 and IL-13 on osteoclast differentiation from BMMs (osteoclast precursors) using a BMM culture system. As shown in Fig. 3(a), IL-4 at 3 ng/ml completely inhibited osteoclast differentiation induced by RANKL, while the same concentration of IL-13 demonstrated only slight inhibition and did not completely inhibit osteoclast differentiation even at a concentration of 10 ng/ml. The IC_{50} values of IL-4 and IL-13 for osteoclast differentiation were 0·34 and 13·54 ng/ml, respectively (Figs 3a,b). These results suggest that the stronger inhibitory action of IL-4 observed in our coculture system was caused by the different sensitivities of osteoclast precursors to IL-4 and IL-13.

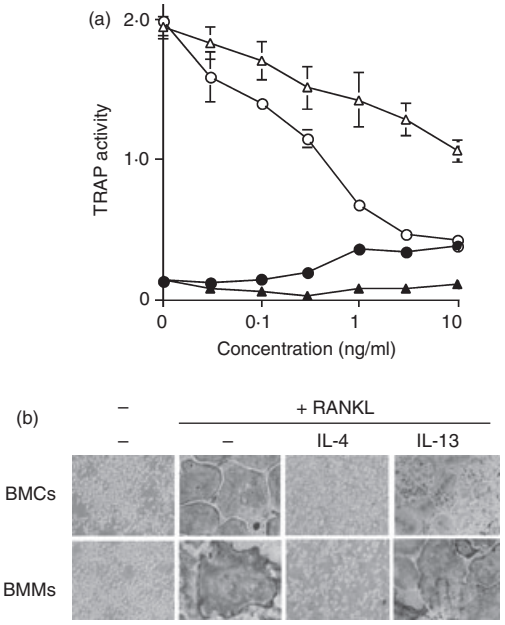

Figure 3. Effects of IL-4 and IL-13 on osteoclast differentiation induced by RANKL in osteoclast precursor cultures. (a) BMMs were cultured with various concentrations of IL-4 with (○) or without (●) 150 ng/ml of RANKL, or IL-13 with (△) or without (▲) 150 ng/ml of RANKL. After culturing the cells, the cell lysates were harvested and TRAP activity was measured. Data are shown as the means ± SE ($n = 4$). (b) Photographs of BMCs and BMMs treated with 10 ng/ml of IL-4 or IL-13. The cells were fixed and stained for TRAP (original magnification ×10).

Expression of receptor components for IL-4 and IL-13 in osteoclast precursors and osteoblasts

To explore the mechanism of IL-4 and IL-13 action, we analysed the expressions of receptor components for IL-4 and IL-13 (Fig. 4). Although IL-4Rα and IL-13Rα1 mRNAs were commonly expressed in all the cells tested, the γc mRNA was expressed in osteoclast precursors such as BMCs and BMMs but not in osteoblastic cells such as primary osteoblasts and UAMS-32 cells (Fig. 4). Consistently, the RAW264.7 cell line known as osteoclast precursors and the ST-2 stromal cell line showed the same expression patterns with BMCs/BMMs and osteoblasts/UAMS-32 cells, respectively (Fig. 4).

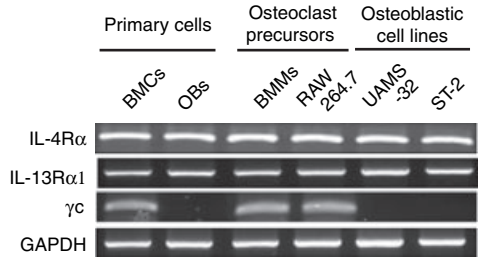

Figure 4. Expression of receptor components for IL-4 and IL-13 in osteoclast precursors and osteoblastic cells. Total RNAs were extracted from BMCs, primary osteoblasts (OBs), BMMs, RAW264.7 cells, UAMS-32 cells, and ST-2 cells. Subsequently, the mRNA expressions of IL-4Rα, IL-13Rα1, γc, and GAPDH in the cells were analysed by RT–PCR.

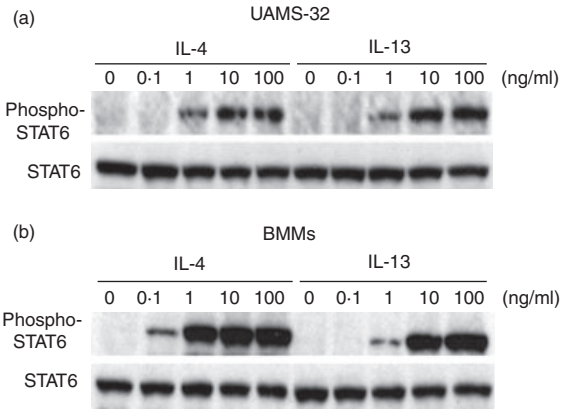

Figure 5. Phosphorylation of STAT6 by IL-4 and IL-13 in UAMS-32 cells and BMMs. UAMS-32 (a) cells and BMMs (b) were treated with 0·1, 1, 10 and 10 ng/ml of IL-4 and IL-13 for 30 min. Subsequently, phosphorylation of STAT6 protein was analysed by Western blot analysis.

Activation of STAT6 by IL-4 and IL-13 in osteoclast precursors and osteoblasts

In the UAMS-32 cells, phosphorylation of STAT6 occurred in the presence of as little as 1 ng/ml IL-4 or IL-13 and increased in a dose-dependent manner, with phosphorylation levelling off at 10 ng/ml (Fig. 5a). On the other hand, in the BMMs, phosphorylation of STAT6 occurred at lower concentrations of IL-4 ($\geq$0·1 ng/ml) than those of IL-13 ($\geq$ 1 ng/ml) (Fig. 5b). These results suggest that the effects of IL-4 and IL-13 on the activation of STAT6 in osteoblasts are equivalent, but IL-4 proved to be more effective than IL-13 for activation of STAT6 in osteoclast precursors.

Effects of IL-4 and IL-13 on expression of NFATc1 mRNA in osteoclast precursors

Because NFATc1 is known to be a critical gene for regulating the differentiation of osteoclasts,[19] we examined the effects of IL-4 and IL-13 on the expression of NFATc1 mRNA in BMMs treated with RANKL (Fig. 6). The expression of NFATc1 mRNA induced by RANKL in BMMs was completely inhibited by 10 ng/ml of IL-4, while the same concentration of IL-13 demonstrated only slight inhibition. This is consistent with the results observed in the BMM culture system following treatment with IL-4 and IL-13.

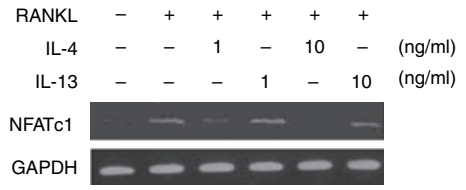

Figure 6. Effects of IL-4 and IL-13 on NFATc1 mRNA expression in osteoclast precursors. BMMs were treated with or without 150 ng/ml of RANKL in the presence of IL-4 or IL-13 at the indicated concentrations for 24 hr. Total RNA was then extracted and NFATc1 mRNA expression was examined using RT–PCR.

Discussion

It has been reported that IL-4 and IL-13 inhibit osteoclast differentiation in a similar manner.[14,20–24] However, the present results demonstrated some differences in regard to their activities toward osteoclast precursors and osteoblasts. Our data suggest that the inhibitory effect of IL-4 is stronger than that of IL-13 toward osteoclast precursors, while their effects on OPG and RANKL mRNA expression in osteoblasts are equivalent.

After comparing the IC_{50} values of IL-4 and IL-13 between the coculture and BMM culture systems, it was apparent that both cytokines inhibited osteoclast differentiation more strongly in the coculture system than in the BMM culture system, as their IC_{50} values were 0·13 ng/ml and 2·69 ng/ml, respectively, in the coculture system, and 0·34 ng/ml and 13·54 ng/ml, respectively, in the BMM culture system. These results suggest that inhibition of osteoclast differentiation by IL-4 and IL-13 is derived from not only their direct actions on osteoclast precursors, but also indirect actions on osteoblasts, including up-regulation of OPG production and down-regulation of RANKL expression.

Palmqvist *et al.* showed that IL-4 and IL-13 at a concentration of 50 ng/ml slightly increased OPG mRNA expression levels in calvarial osteoblasts after 48 hr, when evaluated using RT–PCR.[14] Our time course and dose–response results using Northern blot analysis indicated that the cytokines strongly induced OPG mRNA expression after 1 hr, even at 1 ng/ml. Further, the maximum expression levels of OPG mRNA were observed at 3 hr and decreased thereafter. These results suggest that OPG production is regulated very quickly and strongly by stimulation with IL-4 and IL-13. As for RANKL, a concentration of 1 ng/ml of IL-4 or IL-13 was enough to down-regulate its expression for 24 hr. Thus, IL-4 and IL-13 may regulate OPG and RANKL production in osteoblasts to inhibit osteoclast differentiation in a manner more sensitive than previously supposed.

In the immune system, IL-4 is necessary for $CD4^+$ Th2 cell development, while IL-13 has been reported to have negligible effects on T cells.[25] These functional differences have been explained by the different expression patterns of the receptor subunits.[26] The present results showed that IL-4Rα and IL-13Rα1 are commonly expressed in osteoclast precursors and osteoblasts, while γc is expressed in osteoclast precursors but not in osteoblasts. Palmqvist *et al.* showed that the expression of IL-13Rα2, a decoy receptor for IL-13 signalling, is decreased by treatment with RANKL and M-CSF in BMMs, and recovered by IL-13.[14] Therefore, the expression patterns of the receptor subunits may be related, at least in part, to the inhibitory actions of IL-4 and IL-13 toward osteoclast differentiation.

Moreno *et al.* showed that IC_{50} for IL-4 induced suppression of osteoclast differentiation correlated directly to the EC_{50} for inducing the tyrosine phosphorylation of STAT6.[13] We further revealed that IL-4 induces tyrosine phosphorylation of STAT6 at lower concentrations than IL-13 in osteoclast precursors, while that in osteoblasts was equivalent. These results are consistent with those observed in the analysis of osteoclast differentiation, OPG production and NFATc1 expression. Thus, levels of STAT6 activation may be crucially involved in the inhibition of osteoclast differentiation. Further investigation including the analysis of JAK3 phosphorylation is required to reveal the precise roles of intracellular signalling pathways in the regulation of osteoclast differentiation.

Osteoclast precursors are multipotential cells that differentiate into immune cells, such as mature macrophages and dendritic cells.[9,28] Although NFATc1 has been reported to regulate osteoclast differentiation induced by RANKL, the factor is also involved in immune cell functions.[29] Our RT–PCR analysis showed that a concentration of 10 ng/ml of IL-4, but not IL-13, inhibited NFATc1 expression in osteoclast precursors. Therefore, we concluded that IL-4 and IL-13 differently regulate the balance of differentiation into immune cells and osteoclasts via NFATc1.

Recently, Ahn *et al.* reported that IL-4 was more effective than IL-13 for *in vitro* differentiation of dendritic cells from peripheral blood mononuclear cells.[30] We also found that the inhibitory effects of IL-4 and IL-13 are different toward osteoclast precursors, while they are equivalent toward osteoblasts for the induction of OPG production. Together, our results imply that IL-4 and IL-13 play different roles in bone metabolism, including inhibition of bone resorption.

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